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Transformation of, and Heterologous Protein Expression in, *Lactobacillus agilis* and *Lactobacillus vaginalis* Isolates from the Chicken Gastrointestinal Tract[▽]

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Lactobacilli are naturally found in the gastrointestinal tract of chickens, and there is interest in utilizing autochthonous strains for the delivery of therapeutic proteins. Previously we identified three chicken-derived *Lactobacillus* strains, *Lactobacillus agilis* La3, *Lactobacillus vaginalis* Lv5, and *Lactobacillus crispatus* Lc9, which persist in the gastrointestinal tract of chickens fed either a commercial or high-protein diet. In the current study, we investigated the ability to electrotransform these strains, determined plasmid vector stability, and compared reporter gene expression directed by several different promoters. The La3 and Lv5 strains were reproducibly transformed with efficiencies of 10^8 and 10^6 transformants per microgram of plasmid DNA, respectively. The third strain tested, *L. crispatus* Lc9, was recalcitrant to all transformation protocols examined. The plasmid vectors pTRK563 and pTRKH2 were maintained over 100 generations in La3 and Lv5, respectively. The ability of La3 and Lv5 to express the heterologous reporter gene *gfp* was analyzed using heterologous and homologous promoters. Transformants of both La3 and Lv5 containing the La3 *ldhL* promoter were the most fluorescent. To our knowledge, this is the first report of successful transformation and heterologous protein expression in *L. agilis* and *L. vaginalis*. The ability of these strains to express heterologous proteins *in vitro* indicates their potential utility as *in vivo* delivery vectors for therapeutic peptides to the chicken gastrointestinal tract.

Lactobacilli are autochthonous inhabitants of the chicken gastrointestinal tract (GIT), where they predominate the upper GIT microbiota (53). Their dominance of the upper GIT makes lactobacilli excellent candidates for development as live vectors for the delivery of therapeutic proteins targeting bacterial pathogens such as *Clostridium perfringens*, *Campylobacter jejuni*, *Listeria monocytogenes*, or *Salmonella enterica.* Additionally, lactobacillus delivery vectors could be used to immunize against avian viruses, such as infectious bursal disease, Marek's disease, Newcastle disease, or avian influenza virus. We have recently identified several *Lactobacillus* strains, *Lactobacillus agilis* La3, *Lactobacillus vaginalis* Lv5, and *Lactobacillus crispatus* Lc9, which are able to colonize and persist within the GIT of chickens fed either a commercial or a high-protein diet (45). These strains show great potential as vectors for the *in situ* delivery of therapeutic proteins targeting GIT pathogens.

Lactobacilli have several advantages as mucosal delivery vectors (54), including "generally regarded as safe" status, survival within the GIT, stimulation of immune responses in the mucosa, and the potential to be engineered to express therapeutic peptides. In regard to the latter, lactobacilli are notoriously recalcitrant to transformation, representing an important limitation in realizing the vectoring potential of persistent strains. Several studies have investigated the genetic transformation of chicken *Lactobacillus* strains (5, 28, 43), identifying some strains that could be transformed while others remained recalcitrant. To our knowledge, transformation of the species *L. agilis* and *L. vaginalis* has not previously been reported, while strain-dependent transformation has been described in *L. crispatus* (5, 28) along with *Lactobacillus salivarius* (28, 50) and *Lactobacillus reuteri* (1, 26, 55), which are phylogenetically related to *L. agilis* and *L. vaginalis* (17), respectively.

Strains intended for use as delivery vectors must be able to express genes of interest *in vivo*. A key factor in high-level gene expression is the selection of an appropriate and effective transcriptional promoter. Inducible promoters have been investigated for heterologous gene expression in lactobacilli, particularly the nisin-controlled expression system (13, 41, 55). Previous studies examining the use of the nisin-inducible system *in vivo* have used cultures induced *in vitro* prior to administration (13, 33). Limitations of utilizing this inducible expression system *in vivo* include degradation of nisin within the GIT (6, 37), although low levels may be sufficient to induce gene expression *in vivo* (37), and increased costs incurred from the addition of the inducer to food or water. Native regulatory systems naturally inducible *in vivo*, or constitutive promoters, could ensure constant production of therapeutic peptides at the site of colonization. Several constitutive promoters have previously been used for heterologous expression in lactobacilli, including the P_{ldhL} (20, 26), P_{slpA} (7, 26, 29, 31), P_{144} (15), and lactococcal P_{23} (10) promoters. Recently, several groups have described the use of homologous promoters for highly efficient gene expression (11, 16, 30). These studies highlight

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid[a]	Origin or relevant characteristic(s)	Source and/or reference(s)
Bacterial strains		
Lactobacillus agilis La3	Chicken jejunum, persists in chicken GIT	44 and 45
Lactobacillus crispatus Lc9	Chicken jejunum, persists in chicken GIT	44 and 45
Lactobacillus vaginalis Lv5	Chicken crop, persists in chicken GIT	44 and 45
E. coli JM109	*endA1 recA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44* Δ(*lac-proAB*) [F′ *traD36 proAB laqI*^{q}ZΔM15]	Promega
Plasmids		
pTRKH2	Erm^r, high-copy-no. *E. coli*/ *Lactobacillus* shuttle vector	32
pTRK563	Erm^r, Δ*cat* derivative of pGK12 with *lacZ* from pBluescript II KS(+)	39
pGEM-T Easy	Amp^r, used for initial cloning of homologous promoters	Promega
pGH	Amp^r, used for initial cloning of heterologous promoters	Celtek Genes
pFVP25.1 Addgene plasmid 20668	Amp^r, source of *gfp* gene	Addgene
pTRKH2:P_{slpA}:*gfp*:T_{slpA} (het)	pTRKH2 with *slpA* promoter, *gfp* gene, *slpA* terminator	This study
pTRKH2:P_{23}:*gfp*:T_{Laf} (het)	pTRKH2 with P23 promoter, *gfp* gene, *Laf* terminator	This study
pTRKH2:P_{144}:*gfp*:T_{T7} (het)	pTRKH2 with P144 promoter, *gfp* gene, T7 terminator	This study
pTRKH2:P_{cysK}:*gfp*:T_{slpA} (hom)	pTRKH2 with *cysK* promoter, *gfp* gene, *slpA* terminator	This study
pTRKH2:P_{ldhL}:*gfp*:T_{slpA} (hom)	pTRKH2 with *ldhL* promoter, *gfp* gene, *slpA* terminator	This study
pTRKH2:P_{pgm}:*gfp*:T_{slpA} (hom)	pTRKH2 with *pgm* promoter, *gfp* gene, *slpA* terminator	This study
pTRKH2:P_{hlb}:*gfp*:T_{slpA} (hom)	pTRKH2 with *hlb* promoter, *gfp* gene, *slpA* terminator	This study

[a] The promoters have been designated heterologous (het) and homologous (hom) with respect to La3; all promoters are heterologous in Lv5.

the advantages of using native promoters, as the endogenous transcriptional signals are guaranteed to be recognized by the host strain. Advances in genome sequencing facilitate fast and efficient identification of native promoters. An easily detectable reporter gene, such as the green fluorescence protein (GFP) gene of *Aequorea victoria*, has previously been used to monitor heterologous expression in lactobacilli (13, 16, 26, 55, 56). The use of GFP as a reporter has several advantages, including ease of detection without the need for substrate addition and simple quantification of expression by fluorometry.

The primary aims of the current study were to develop and optimize a protocol to transform *L. agilis* La3, subsequently investigate plasmid stability, and compare heterologous versus homologous promoters for the expression of GFP. Transformation of other candidate strains, *L. vaginalis* Lv5 and *L. crispatus* Lc9, was also investigated, as were plasmid stability and GFP expression in *L. vaginalis*. Here, we report the first successful transformation of *L. agilis* and *L. vaginalis* isolates. Comparison of heterologous and homologous promoters revealed that the *L. agilis* La3 *ldhL* promoter produced the highest levels of GFP expression within the *L. agilis* and *L. vaginalis* strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains used in this study are listed in Table 1. *Lactobacillus agilis* La3 and *L. crispatus* Lc9 were originally isolated from the jejuna and *L. vaginalis* Lv5 was isolated from the crops of chickens fed an experimental high-protein diet (44) and subsequently found to persist in the chicken GIT upon reinoculation (45). Lactobacilli were routinely cultured in MRS broth (Difco, Detroit, MI) and agar at 37°C for 24 and 48 h, respectively, under anaerobic conditions using a BD GasPAK EZ container (Becton Dickinson, Sydney, Australia) with an AnaeroGen sachet (Oxoid Australia, Adelaide, Australia). *Escherichia coli* was routinely cultured in Luria-Bertani (LB) (40) broth and agar at 37°C for 16 to 24 h. Concentrated stocks of the antibiotic erythromycin (Erm) were prepared as described by Sambrook and Russell (40) and used at a concentration of 15 μg ml^{-1} for *Lactobacillus* (MRS_{Erm15}) and 250 μg ml^{-1} for *E. coli* (LB_{Erm250}). The growth of the *Lactobacillus* strains was assessed in MRS media containing glycine concentrations ranging from 0 to 2.5% (wt/vol).

DNA isolation and manipulations. Genomic DNA from *Lactobacillus* strains was isolated using the method described previously (44). Plasmid DNA was isolated from *E. coli* using the Qiagen plasmid midikit (Qiagen Pty Ltd., Melbourne, Australia) for large-scale preparations, while small-scale preparations were performed using the Wizard Plus SV minipreps DNA purification system (Promega, Sydney, Australia). Plasmid DNA was isolated from *Lactobacillus* isolates using the protocol of Walker and Klaenhammer (52), with the exception that 1.8 M acetic acid was used instead of 1.8 M formic acid prior to the phenol chloroform extraction step. PCR purification was performed using the Wizard SV gel and PCR cleanup system (Promega). Amplified PCR products were cloned into pGEM-T Easy vector system I (Promega) according to the manufacturer's instructions. Restriction enzymes and T4 DNA ligase (New England Biolabs, Ipswich, MA) were used according to the manufacturer's directions.

Genetic transformation. Chemically competent *E. coli* JM109 (Promega) was transformed according to the manufacturer's directions. Lactobacilli were transformed by electroporation using the methods described in Table 2. Electroporation was routinely carried out in 0.2-cm electroporation cuvettes (Bio-Rad, Gladesville, Sydney, Australia) within a Gene Pulser II apparatus (Bio-Rad) using 3 μg of plasmid DNA (0.6 to 5 μl). After the expression period (Table 2), the transformants (TF) were plated onto MRS_{Erm15} agar plates and incubated for 48 to 72 h. The transformation efficiency was calculated as the number of transformants per microgram of plasmid DNA (TF μg plasmid DNA^{-1}).

Plasmid stability. The *in vitro* stability of pTRKH2 and pTRK563 was assessed within *L. agilis* La3 and *L. vaginalis* Lv5 by culturing transformants in MRS broth without Erm for 100 generations. For each transformant, the number of generations in a 24-h culture was determined, followed by daily transfer into fresh MRS broth without Erm until 100 generations was achieved. Subsequently, the cultures were plated on MRS agar and incubated as described above, and 100 colonies were patched onto MRS and MRS_{Erm15} agar plates. The percentage of plasmid stability was determined as the percentage of Erm-resistant colonies relative to the total number of viable colonies. The plasmid stability was assessed using three different transformants for each strain. The percentage of plasmid stability was averaged for all three transformants, with the mean and standard error of the mean reported.

Generation of GFP expression constructs. The heterologous promoters and terminators (Table 3) were synthesized by Celtek Genes (Celtek Bioscience, Nashville, TN) in pGH and transformed into *E. coli* JM109.

A draft genome sequence of the *L. agilis* La3 strain was generated using high-throughput pyrosequencing technology (Roche/454 Life Sciences, Branford, CT) and analyzed to identify promoters for use in homologous expression constructs (Table 3). Identification of the genes, and corresponding promoters, within the draft *L. agilis* La3 genome was performed by using BLAST (3) to align

TABLE 2. Protocols used to transform *L. agilis* La3

Source of transformation protocol	Cell growth conditions	Cell wash conditions	Electroporation parameters	Dilution, incubation temp, and expression time	Transformation efficiency using pTRK563 (TF μg plasmid DNA^{-1})
Luchansky et al. (27) (original)	Overnight culture used as 1/100 inoculum into fresh MRS broth. Incubated statically at 37°C until the OD_{590} reached 0.6.	Cells were harvested by centrifugation ($8,000 \times g$) at 4°C and washed four times in ice-cold SMEB (1 M sucrose, 25 mM $MgCl_2$ electroporation buffer) and concentrated to 100 times the original culture vol.	2.5 kV, 200 Ω, 25 μF	Cells were diluted 1/5 in MRS containing 0.3 M sucrose and incubated at 37°C for 3 h.	$0–1.60 \times 10^4$
Luchansky et al. (27) (optimized for La3)	Overnight culture was inoculated 1/100 into MRS containing 0.5% glycine (wt/vol) and incubated until the OD_{590} reached 0.6.	As above, except cells were concentrated to 50 times the original culture vol.	2.0 kV, 200 Ω, 25 μF	Cells were diluted 1/5 in MRS broth and incubated at 37°C for 3 h.	$1.94 \times 10^4–8.28 \times 10^4$
Mason et al. (28)	Overnight cultures were used as 1/6 inoculum in MRS broth (12 ml) containing 2% glycine (wt/vol). Cultures were incubated at 37°C 90 min.	Cells were harvested by centrifugation ($8,000 \times g$) at 4°C and washed twice in 1 ml ice-cold ultrapure (Milli-Q) water. Cells were resuspended in 1 ml 0.5 M EDTA and incubated on ice for 5 min. Cells were washed twice in 1 ml 0.3 M sucrose prior to resuspension in 100 μl 0.3 M sucrose (120 times the original cell concn).	1.5 kV, 200 Ω, 25 μF	Cells were immediately diluted 1/20 in MRS broth prewarmed to 37°C, followed by incubation at 37°C for 3 h.	$1.83 \times 10^7–2.87 \times 10^8$

the *L. agilis* La3 genome scaffolds with the sequences of constitutively expressed genes from other lactobacilli (Table 3). Putative promoter sequences upstream of these La3 genes were identified by searching for −35 and −10 region consensus sequences described previously (35). These consensus sequences were also predicted using the Web-based program Prokaryotic Promoter Prediction (PPP) (http://bioinformatics.biol.rug.nl/websoftware/ppp/). PCR primers were designed to amplify regions upstream of the −35 region and downstream of the −10 region along with the relevant restriction sites required for cloning (Table 4). Genomic DNA of *L. agilis* La3 was employed as the template for PCR amplification of the homologous promoters P_{cysK}, P_{ldhL}, P_{pgm}, and P_{hlb}, using the primers and PCR protocols outlined in Table 4. The PCR products were ligated into the pGEM-T Easy vector system and transformed into *E. coli* JM109 as described above. The sequences of the different promoters were compared using a Clustal W alignment (46).

A promoterless *gfp* construct in pTRKH2 was used as the base plasmid into which homologous and heterologous promoters were inserted. The plasmid pTRKH2::*gfp*:T_{slpA} was generated by digestion of pTRKH2 with XbaI and BamHI, followed by ligation with the synthesized *slpA* terminator in pGH, which had been digested with SalI and BamHI. The *gfp* gene, amplified from the plasmid pFVP25.1 (Addgene plasmid 20668; Addgene Inc., Cambridge, MA) by using the primers described in Table 4, was digested with BglII and SalI and ligated into the pTRKH2:::T_{slpA} plasmid, resulting in pTRKH2::*gfp*:T_{slpA}. Plasmid DNAs containing the heterologous and homologous promoters in pGH and pGEM-T Easy, respectively, were extracted from the *E. coli* JM109 transformants, digested with XbaI and BglII, and cloned into the promoterless pTRKH2::*gfp*:T_{slpA} plasmid digested with the same enzymes. The integrity of the obtained transformants was verified by PCR, using primers M13Fwd (located downstream of the multiple-cloning site [MCS] in pTRKH2::*gfp*:T_{slpA}) and the forward primer for each promoter (located on the insert). The various expression plasmids that resulted are listed in Table 1.

Detection of GFP expression. GFP expression in *Lactobacillus* was achieved using the protocol of Wu and Chung (55). Briefly, an overnight culture was subcultured 1/50 into MRS_{Erm15} broth and incubated statically at 37°C for 3 h, followed by 3 h at 28°C with 250 rpm shaking. The cultures were pelleted and washed twice in 500 μl phosphate-buffered saline (PBS; pH 7.2; Gibco, Invitrogen). The cultures were diluted to an optical density at 590 nm (OD_{590}) of 0.3 in PBS and were examined by fluorometry and microscopy. The cultures (200 μl of resuspended cells) were pipetted into a 96-well flat-bottom plate, and the fluorescence was measured using a FLUOstar Optima plate reader (BMG Labtech, Mornington, VIC, Australia). The gain was set on the most fluorescent sample, from which the relative number of fluorescent units (RFU) was determined. The number of RFU in the blank, PBS only, was subtracted from the number of RFU in the sample. The averages and standard errors of the means from three independent experiments were determined. Fluorescent microscopy was performed by pipetting 10 μl of resuspended cells onto a glass slide onto which a coverslip was placed, and cells were viewed under the oil immersion lens of a Leica DM LB microscope (Leica Microsystems, North Ryde, Sydney, Australia) equipped with a fluorescence filter (Leica filter cube H3) and a mercury lamp (ebq 100 dc-1 [100 W]; LEJ, Jena, Germany). Images were captured using an RT slider (SPOT Image Solutions, Diagnostic Instruments Inc., Sterling Heights, MI) and SPOT software (v4.09; SPOT Image Solutions).

Nucleotide sequence accession numbers. The GenBank accession numbers of the La3 *cysK*, *ldhL*, *pgm*, and *hlb* promoters and corresponding genes identified in this study are HQ435310, HQ435311, HQ435312, and HQ435313, respectively.

RESULTS

Transformation of *L. agilis* La3, *L. crispatus* Lc9, and *L. vaginalis* Lv5. Initially, several protocols were tested (Aukrust et al. [4], van Pijkeren et al. [50], and Luchansky et al. [27]) for the transformation of *L. agilis* La3. Only the Luchansky protocol resulted in sporadic low-level transformation efficiencies (Table 2), so attempts were made to optimize the protocol specifically to this strain. The La3 strain was assessed for its ability to grow in MRS broth containing various concentrations of glycine (data not shown), as glycine has been shown to increase lactobacillus transformation efficiencies (43). MRS containing up to 0.5% glycine (wt/vol) only slightly reduced growth efficiency compared to that of MRS only. MRS con-

TABLE 3. Regulatory signals used for heterologous/homologous expression in *L. agilis* La3 and *L. vaginalis* Lv5

Signal	Source	Relevant characteristic	GenBank accession no. (bp used)	Reference	Reference(s) showing example of previous use in heterologous expression
Heterologous signals					
slpA promoter	*Lactobacillus acidophilus* ATCC 4356	Surface-layer protein constitutive promoter	X89375 (7–262)	8	7, 29, 31
P_{23} promoter	*Lactococcus lactis* subsp. *cremoris* Wg2	Promoter 23	M24763 (7–127)	49	10, 24, 49
P_{144} promoter	*Lactobacillus paracasei* CG11	Promoter-like sequence 144	S76789 (7–96)	15	15
amyA RBS[a]	*Lactobacillus amylovorus* NRRLB4540	Alpha-amylase RBS	X80271 (108–126)	18	
Sequences used to identify homologous signals in the La3 genome					
cysK promoter	*L. salivarius* UCC118	Cysteine synthase constitutive promoter	NC_007929 (1797853–1798162)	12	16
ldhL promoter	*Lactobacillus casei* ATCC 393	L-Lactate dehydrogenase promoter	M76708 (7–141)	23	31, 36
hlb promoter	*Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842	Histone-like DNA-binding protein promoter	NC_008054 (740999-741274)	48	11
pgm promoter	*L. acidophilus* NCFM	Phosphoglyceromutase promoter	NC_006814 (178473–179177)	2	30

[a] RBS, ribosome binding site.

taining glycine concentrations of >0.5% (wt/vol) markedly reduced the growth rate, while concentrations of ≥0.8% completely inhibited the growth of La3. Incorporation of 0.5% glycine (wt/vol) into the modified Luchansky protocol resulted in consistent transformation efficiencies (Table 2). To determine if higher glycine concentrations could further improve transformation efficiency, the protocol of Mason et al. (28) was tested, whereby a glycine "pulse" was applied to the strain. The pulse consisted of diluting a stationary-phase culture 1/6 into MRS containing 2% glycine (wt/vol) and incubat-

TABLE 4. PCR primers used in this study

Primer name[a]	Primer sequence 5′ to 3′[b]	Target (GenBank accession no.)	Annealing temp dropdown (°C)[c]	Source
M13 Fwd	GTAAAACGACGGCCAGT	MCS of pTRKH2 and pTRK563	56–50	pBluescript KS(+); Stratagene, La Jolla, CA
M13 Rev	GGAAACAGCTATGACCATG	MCS of pTRKH2 and pTRK563		Stratagene
GFP-1Fwd	GTGCCTAAAGATCT**ATAAAGGGGGCAGTAAA AA**ATGAGTAAAGGAGAAGAACTTTTCAC	*amyA* RBS and mRNA start codon translationally fused to the *gfp* gene	70–60	This study
GFP-1Rev	GGGCTTTTGTCGACTTATTTGTAGAGCTCA TCCATGCC	*gfp* gene		This study
La3 cysKp Fwd	CCCCCCCCTCTAGATGAAGACCGGATGCAA ATTAC	*L. agilis* La3 P_{cysK} (HQ435310)	56–50	This study
La3 cysKp Rev	CCCCCCCCAGATCTAATTAATCTGCTTACTT TTTG	*L. agilis* La3 P_{cysK} (HQ435310)		This study
La3 ldhLp Fwd	CCCCCCCCTCTAGACCTCTTTAACTAACAG CGCC	*L. agilis* La3 P_{ldhL} (HQ435311)	65–55	This study
La3 ldhLp Rev	CCCCCCCCAGATCTCTTATTGATACAAATAT ATTATACC	*L. agilis* La3 P_{ldhL} (HQ435311)		This study
La3 pgmp Fwd	CCCCCCCCTCTAGATGATTTAGAGGGAACT TACCTAATGTG	*L. agilis* La3 P_{pgm} (HQ435312)	70–60	This study
La3 pgmp Rev	CCCCCCCCAGATCTAATTGTTTACACCTGTA TTTTACAC	*L. agilis* La3 P_{pgm} (HQ435312)		This study
La3 hlbp Fwd	CCCCCCCCTCTAGAATTTTCATGTGATCGTT TTCTTTTG	*L. agilis* La3 P_{hlb} (HQ435313)	65–55	This study
La3 hlbp Rev	CCCCCCCCAGATCTTCCGCACGAGTTGAAA CAATG	*L. agilis* La3 P_{hlb} (HQ435313)		This study

[a] Fwd and Rev indicate forward and reverse primers, respectively.
[b] Underlining indicates a restriction enzyme site, while bolding indicates the *amyA* RBS and untranslated leader sequence.
[c] PCR programs each used an initial melting cycle of 94°C for 3 min, followed by 2 cycles of 94°C for 30 s, dropdown temperature 1 (e.g., 56°C) for 45 s, and 72°C for 1 min, 2 cycles of the following dropdown temperature, and so on until the final annealing temperature (e.g., 50°C), at which point 30 cycles were performed, followed by a final elongation of 72°C for 5 min.

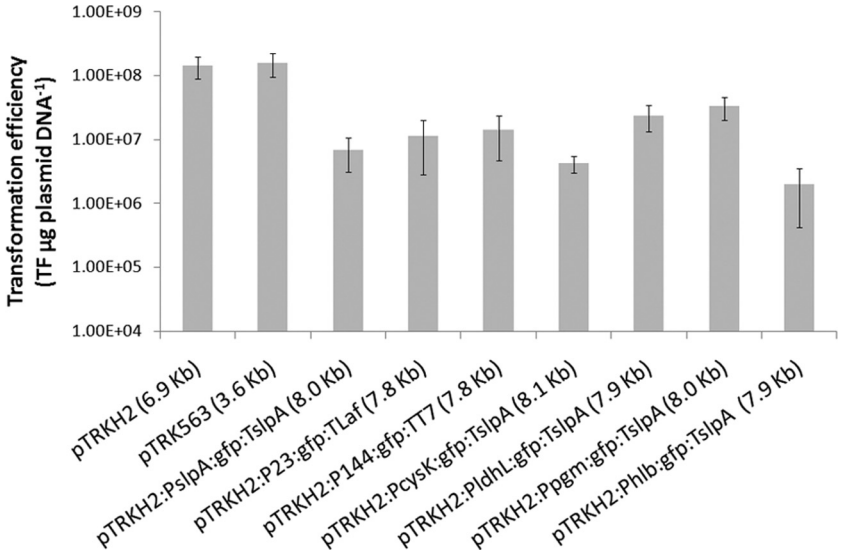

FIG. 1. Transformation efficiency of *L. agilis* La3 using various plasmids and the Mason et al. transformation protocol. Plasmid pTRKH2 and derivatives replicate via the theta mechanism, while pTRK563 replicates via the sigma mechanism. The transformation data presented are from at least three replicates of three independent transformants and represent the mean transformation efficiencies, with the standard errors of the means incorporated as error bars.

ing for 90 min at 37°C and had little effect on the viability of the cells. The glycine and/or other parameters of the Mason protocol, such as growth phase or cell wash conditions, resulted in an increased transformation efficiency of 4 orders of magnitude (Table 2).

A similar approach was used in the investigation of *L. crispatus* Lc9 and *L. vaginalis* Lv5 transformation efficiencies. Analogous to La3, transformation of Lv5 was unsuccessful using the Aukrust and van Pijkeren protocols and was only sporadically successful using the Luchansky protocol. Transformation efficiencies of 10^6 TF μg plasmid DNA^{-1} were achievable with pTRK563 by using the optimized Luchansky protocol with 0.5% glycine (wt/vol) (data not shown), while the Mason protocol resulted in transformation efficiencies of 10^4 to 10^5 TF μg plasmid DNA^{-1} (data not shown). Lc9 was recalcitrant to all attempted transformation protocols mentioned above.

In addition to the increased transformation efficiency of La3, the Mason protocol was less laborious than the Luchansky protocol and was therefore used for all subsequent transformations of the La3 and Lv5 strains.

Comparison of transformation efficiencies by using different plasmids. The effects of plasmid size and mode of replication on the transformation efficiency of La3 were compared (Fig. 1). Plasmids pTRKH2 and pTRK563 had similar efficiencies (1.43×10^8 and 1.58×10^8 TF μg plasmid DNA^{-1}, respectively) even though they have different replication mechanisms (theta and sigma replication, respectively) and sizes (6.9 kb and 3.6 kb, respectively). Based upon restriction sites within the multiple cloning sites of pTRKH2 and pTRK563, the former was selected for use in the subsequent expression experiments. When the expression cassettes were cloned into pTRKH2 and introduced into La3, the transformation efficiency dropped by 10^1 to 10^2 TF μg plasmid DNA^{-1} (Fig. 1). These results indicate that regardless of the mechanism of plasmid replication and size, transformation efficiencies of $>10^6$ TF μg plasmid DNA^{-1} were routinely observed for La3 using the Mason protocol.

The *in vitro* plasmid stability after 100 generations without selective pressure (Erm) in La3 was 3.3% (±2.4% [standard error of the mean]) and 98.0% (±1.0%) for pTRKH2 and pTRK563, respectively, while in *L. vaginalis* Lv5 the stability of pTRKH2 and pTRK563 was 98.7% (±1.3%) and 8.7% (±2.3%), respectively.

Heterologous expression of GFP using heterologous and homologous promoters. The GFP expression plasmids (Table 1) with different regulatory signals (Tables 3 and 4) were constructed and initially propagated within *E. coli*. The heterologous promoters were tested first for their ability to direct expression of GFP. Each promoter was constitutively active within *E. coli*, resulting in the expression of GFP (data not shown), confirming the integrity of the constructs. The plasmids were extracted, purified, and transformed into La3 and Lv5. GFP expression was analyzed by fluorometry, and three individual transformants of each strain were investigated (Fig. 2A and B). The heterologous promoters produced very little GFP expression within both La3 and Lv5.

Subsequently, putative homologous promoters were identified from a draft version of the La3 genome (Table 4) and cloned into the GFP expression constructs in an effort to improve GFP production. The La3 promoters were constitutively active in *E. coli* (data not shown), like the heterologous promoters, and in La3 and Lv5 (Fig. 2), confirming the biological activity of the predicted promoters. Comparison of the amount of fluorescence emitted from La3 and La5 transformants indicated that each of the La3 promoters directed more GFP production than the heterologous promoters in both strains (Fig. 2A and B). Within La3, the La3 P_{ldhL} promoter produced the highest number of RFU, followed by the P_{pgm}, P_{hlb}, and P_{cysK} promoters. Interestingly, analysis of the La3 P_{ldhL} promoter indicated the presence of a second upstream promoter

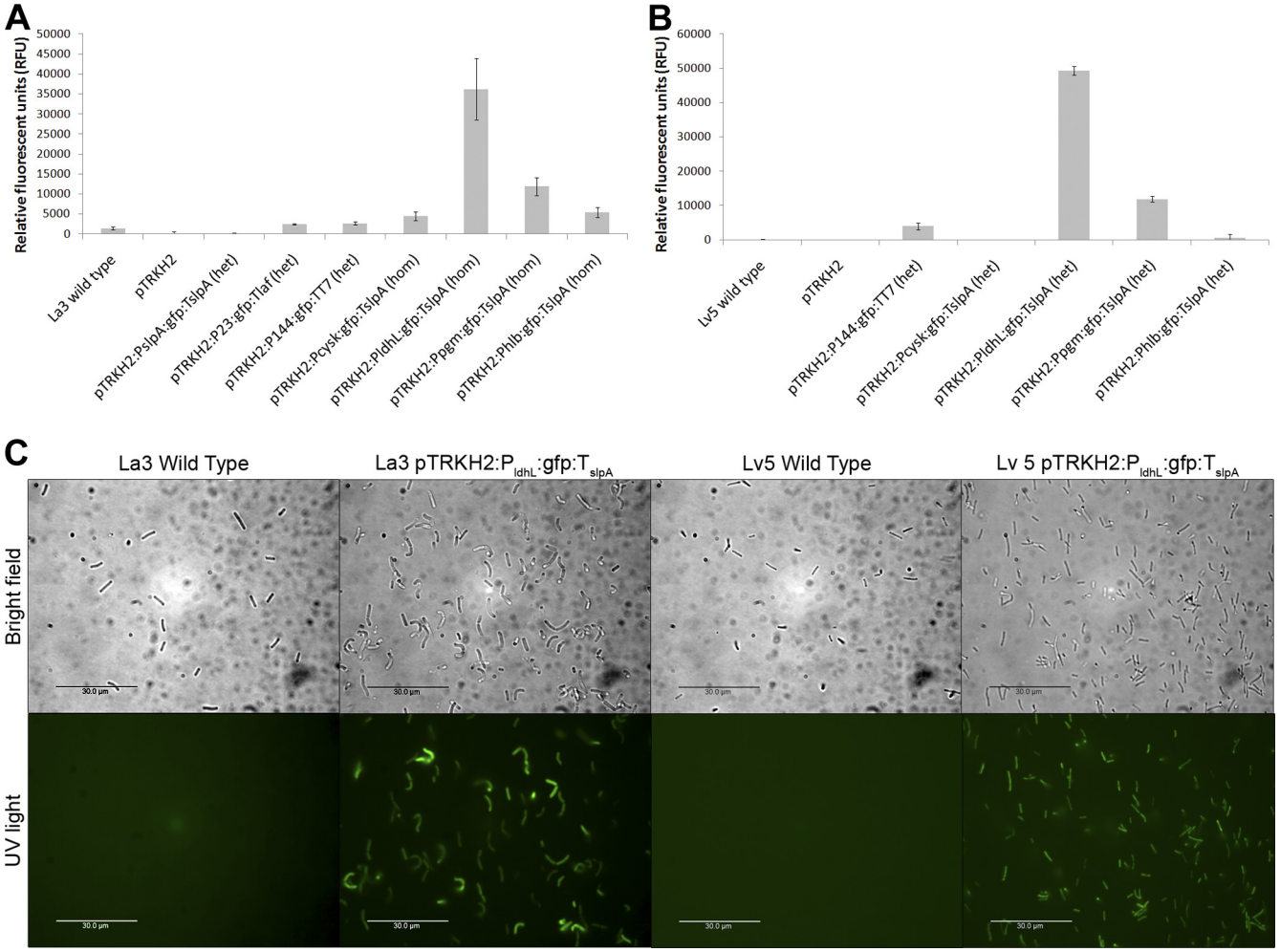

FIG. 2. *L. agilis* La3 and *L. vaginalis* Lv5 GFP expression. Fluorometry results of GFP expression in *L. agilis* La3 (A) and *L. vaginalis* Lv5 (B) using heterologous and homologous promoters, indicated by (het) and (hom), respectively. The data presented are from at least three replicates of three independent transformants and represent the mean numbers of RFU, with the standard errors of the means expressed as error bars. (C) GFP expression in La3 and Lv5 using the pTRKH2:P_{ldhL}:*gfp*:T_{slpA} plasmid, with the wild-type strains shown as negative controls.

region (Fig. 3). GFP production within the La3 transformants containing each of the promoters was confirmed by microscopy (Fig. 2C and data not shown). The fluorometry results indicated that within Lv5, the P_{ldhL} was also the most effective promoter, followed by P_{pgm} and P_{144} (Fig. 2B), and these trends were also confirmed by microscopy (Fig. 2C and data not shown).

DISCUSSION

To our knowledge, these findings represent the first successful and reproducible transformation of the species *L. agilis* and *L. vaginalis*. La3 and Lv5 were transformed using the modified Luchansky and Mason electroporation protocols. The Mason protocol was most efficient for the La3 strain (10^8 TF μg plasmid DNA^{-1}), while the modified Luchansky protocol was more efficient in the Lv5 strain (10^6 TF μg plasmid DNA^{-1}). The *L. crispatus* Lc9 strain was recalcitrant to transformation using the plasmids and protocols applied here, consistent with the literature documenting the recalcitrance of strains belonging to this species (see below). Transformation of La3 was highly efficient when using different plasmid backbones and plasmids containing the insert. Heterologous expression of the reporter gene, *gfp*, was compared within the La3 strain using heterologous and homologous promoters. The identification and subsequent usage of La3 promoters produced higher levels of GFP expression, with P_{ldhL} directing the most GFP production in both the La3 and Lv5 strains. These results demonstrate the ability of these chicken-derived *L. agilis* and *L. vaginalis* strains to express heterologous proteins, which combined with their ability to persist in the chicken GIT (45) makes them excellent candidates as live vectors for the delivery of therapeutic proteins to the GIT of chickens.

Two approaches are typically used to transform natural *Lactobacillus* strains of interest. The first is to take a collection of strains isolated from the area of interest and apply a "one protocol fits all" method. The second is to select strains of interest based upon promising traits, such as their ability to persist, and optimize the transformation protocol in a strain-specific manner. The first approach was used by Beasley et al.

FIG. 3. Clustal W alignment of the homologous and heterologous promoters. The sequences were compared from 50 bp upstream of the −35 hexamer to 13 bp downstream of the −10 hexamer. Putative −35 and −10 hexamers, predicted using the Web-based Prokaryotic Promoter Prediction (PPP) tool, are boxed. The putative mRNA transcriptional start sites are underlined. Consensus nucleotides are in black text, while nonconsensus nucleotides are in gray text. *L. agilis* La3 P_{ldhL} contains a second putative promoter region 11 bp upstream of the promoter, which may be the reason the promoter is most efficient in both *L. agilis* La3 and *L. vaginalis* Lv5.

(5), where only three of the six chicken-derived *L. crispatus* strains were found to be amenable to genetic manipulation. Sieo et al. (43) applied the second approach to several chicken-derived strains (of the species *Lactobacillus acidophilus*, *Lactobacillus brevis*, *L. crispatus*, and *Lactobacillus fermentum*), confirming that each strain required its own optimized protocol. Mason et al. (28) used a mixed approach whereby they optimized a transformation protocol for a single strain, *L. salivarius* Sn1, and then subsequently applied it to another 18 chicken *Lactobacillus* strains, facilitating the transformation of 11 out of 18 previously untransformable strains (including 3/6 *L. crispatus* strains, 5/6 *L. salivarius* strains, 2 *Lactobacillus johnsonii* strains, and 1 *Lactobacillus ingluviei* strain). We also utilized a mixed approach, confirming the results of Sieo et al. (43), whereby each strain was optimally transformed by different protocols. Interestingly, the Mason protocol resulted in a high transformation efficiency with La3 regardless of plasmid replication mechanism, size, or insert. This offers several advantages, as it potentially negates the need for shuttle vectors using intermediate cloning hosts, such as *E. coli*, thus bypassing issues such as instability of A+T-rich sequences, which are common in lactobacilli (17) and difficult to clone in *E. coli* hosts (19), and opens up the possibility of transforming ligations directly. Ligations were used to successfully transform La3 ($\sim 10^4$ TF μg plasmid DNA^{-1}) (data not shown). Furthermore, this high transformation efficiency opens up the possibility of direct homologous recombination into the chromosome by using integration cassettes carried by nonreplicating plasmids, ensuring stability of the integrated DNA without the need for selective markers, such as antibiotic resistance genes. This technique may be particularly advantageous given the poor *in vitro* plasmid stability of pTRKH2 in La3. Integrative cloning strategies require high transformation efficiencies given that integration is a rare event within lactobacilli, as recently examined in *Lactobacillus casei* (14).

The addition of glycine has facilitated higher transformation efficiencies in a variety of lactobacilli (5, 28, 43, 47, 51). Glycine replaces alanine residues within the peptidoglycan precursors in a dose-dependent manner, resulting in weaker cross-linkages in the peptidoglycan layer of the cell (21), making the cells more amenable to electroporation. Our results are consistent with these findings. Growing La3 and Lv5 in subinhibitory concentrations of glycine in the Luchansky protocol resulted in consistent transformation efficiencies in both strains. The Mason protocol increased the transformation efficiency in La3. While it is tempting to speculate that the glycine pulse plays a primary role in this, as indicated in other studies (9, 28), other parameters in the protocol, such as growth phase or cell washes, may have also contributed. Interestingly, the transformation efficiency of Lv5 decreased with the Mason protocol. The lower growth rate of Lv5 (data not shown) may influence the incorporation of glycine into the peptidoglycan during the 90-min pulse. Using the optimized Luchansky protocol, the Lv5 was grown within the 0.5% glycine (wt/vol) MRS broth over many hours and may therefore have been able to incorporate the glycine efficiently into the peptidoglycan layer. An additional study examining differences in the duration, and/or the concentration, of the glycine pulse may improve the Lv5 transformation efficiency.

Clustal W alignment of the promoters used within this study revealed that the promoters are relatively similar. Most have 12 or 13 A and T nucleotides and 4 or 5 G and C nucleotides between the −35 and −10 regions, while the −35 and −10 hexamers differ among the promoters. The *L. agilis* La3 P_{ldhL} promoter has 3 conserved nucleotides in the −35 hexamer (CATACA) compared with the consensus promoter sequence (TTGACA), while the −10 hexamer matches the consensus sequence (TATAAT) (35). One reason the P_{ldhL} may be more active in the La3 and Lv5 strains is an additional putative promoter region 11 bp upstream of the promoter that has −35 and −10 hexamers similar to the consensus sequence (TAGAAA and TAAAAT for the −35 and −10 hexamers, respectively) and may promote more efficient binding of the RNA polymerase. The P_{slpA} promoter of the *L. acidophilus*

ATCC 4356 gene also has two promoter regions which are suspected to help direct efficient expression, although only the downstream promoter directs mRNA synthesis (7). In addition to ensuring high levels of expression, the two −35 and −10 sequences in the La3 P_{ldhL} may facilitate expression under different conditions. Further investigation into the effect of growth phase, *in vivo* conditions, and the role of the duplicate promoter region on the *ldhL* promoter activity is warranted.

In summary, two *Lactobacillus* strains which are able to persist within the chicken GIT under different dietary conditions have been successfully transformed with high efficiency using electroporation. The *L. agilis* La3 *ldhL* promoter produced the most efficient expression of the heterologous reporter gene *gfp* within both La3 and Lv5. Our results are consistent with other studies that have successfully utilized *ldhL* promoters to drive heterologous expression of the *gfp* gene within lactobacilli (20, 26, 56). There are exciting applications in using these strains to deliver proteins to the chicken GIT: either GFP, which could facilitate studies into the mechanism(s) of colonization (34, 56), or therapeutic peptides, such as antigens to be used as mucosal vaccines (54), bacteriocins (38) as an alternative to antibiotics (22), or enzymes to increase production efficiency (25, 42).

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